【別紙２】

論文名：Analysis of the chitin-degrading enzyme system of *Aeromonas salmonicida* and *Serratia plymuthica*

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To develop a novel type of biocontrol agents, in this research we focused on the analysis of the chitin-degrading enzyme system (chitinase system) of *Aeromonas salmonicida* SWSY-1.411 and *Serratia plymuthica* SWSY-3.47, which were previously isolated from a freshwater lake, Sakata, in Niigata.

For chitinase and AA10 protein genes identification, primer pairs were constructed using sequence data of surrounding genes of available sequenced genome of corresponding strains presented in carbohydrate-active enzymes (CAZy) database. The amplified products were cloned and sequenced.

The chitinase system of *A. salmonicida* contains two chitinase genes encoding GH18 chitinases, AsChiA and AsChiB, two chitinase genes encoding GH19 chitinases, AsChiC and AsChiD, and a lytic polysaccharide monooxygenase (LPMO) gene encoding AA10 protein, AsLPMO10A. To characterize these enzymes in detail, the expression and purification systems were constructed. The recombinant proteins were obtained from *Escherichia coli* cells, and the enzymatic properties of these enzymes were analyzed. AsChiA and AsLPMO10A are essential enzymes for synergy, because act synergistically with all chitinases analyzed in the chitinase system of *A. salmonicida*. More than twice increase was observed when powdered chitin was treated by chitinases and AsLPMO10A compared to chitinases combination only. AsChiB is the most powerful chitinase for insoluble chitin degradation, while AsChiD is important for water-soluble chitin degradation. Synergistic effects between chitinases of *A. salmonicida* and known processive chitinases SmChiA or SmChiB of *Serratia marcescens* 2170, and BcChiA1 of *Bacillus circulans* WL-12 have demonstrated that AsChiA has synergy with SmChiA or BcChiA1, that attack the chitin from the reducing end, whereas AsChiB acts synergistically with SmChiB, which degrades the chitin from the non-reducing end. These results had led to suggestion that AsChiA and AsChiB have different role in chitin degradation, and probably digest the chitin from opposite direction. GH19 chitinases, AsChiC and AsChiD, have an important role for antifungal activity in the chitinase system of this strain, and suppress the hyphal growth of *Trichoderma reesei*. According to the amino acid sequence alignment of the catalytic domains, GH19 chitinases of *A. salmonicida* were suggested to be structurally different because have loops II, III, IV, and V in comparison with bacterial GH19 chitinases mainly including *Streptomyces*, which have loops III and IV. Phylogenetically GH19 chitinases of *A.*

salmonicida form the cluster with chitinases of closely related species (such as *Aeromonas*, *Enterobacter*, *Salmonella*, *Vibrio*), which is distantly from bacterial GH19 chitinases mainly including *Streptomyces*. The peroxidase activity of AsLPMO10A was about two times lower compared with that of SmLPMO10A (CBP21) of *S. marcescens* 2170, that could be explained by different domain organization and low (53%) identity between the catalytic domains.

The chitinase system of *S. plymuthica* consists of five chitinase genes encoding GH18 chitinases: SpChiA, SpChiB, SpChiC, SpChiD, and SpChiE, and four LPMO genes encoding AA10 protein: SpLPMO10A, SpLPMO10B, SpLPMO10C, and SpLPMO10D. The chitinase system of *S. plymuthica* was compared with that of well-known chitinolytic bacterium *S. marcescens* 2170. The difference was observed in content of enzymes: the chitinase system of *S. plymuthica* has additional chitinase (SpChiE) and three LPMOs (SpLPMO10A, SpLPMO10B, SpLPMO10C) that lack in the chitinase system of *S. marcescens* 2170. SpChiE was proposed to be an interesting and probably uncharacterized previously chitinase. This enzyme was expressed in *E. coli* cells, and partially purified. SpChiE had high activity to water-soluble chitin and low activity to insoluble chitin. SpChiE did not suppress the hyphal growth of *T. reesei*, and was considered to be like chitinase SmChiC1 of *S. marcescens* 2170 based on the domain organization and obtained enzymatic properties. The role of LPMOs in the chitin-degrading system of *S. plymuthica* was predicted based on the comparison with known LPMOs. Among them, SpLPMO10C was suggested to be uncharacterized LPMO in the chitinase systems of known *Serratia*. Therefore, SpLPMO10C was expressed in *E. coli* cells, and purified. The recombinant SpLPMO10C almost had no synergy with chitinases SpChiB of *S. plymuthica* or SmChiA of *S. marcescens* (used instead of SpChiA of *S. plymuthica*); however, AsLPMO10A (used instead of SpLPMO10A) of *A. salmonicida* and SmLPMO10A (used instead of SpLPMO10D) of *S. marcescens* contributed to synergistic effects with analyzed chitinases, and proposed to be an important for chitin degradation in combination with chitinases.

In this research, we analyzed two different type of chitin-degrading enzyme system of chitinolytic bacteria, *A. salmonicida* and *S. plymuthica*. The model of the chitin-degradation system of *A. salmonicida* was proposed to have an exo-acting processive GH18 chitinases, endo-acting GH19 chitinases (also capable to digest the fungal cell walls), and an important for insoluble chitin depolymerization in conjunction with chitinases AA10 protein. The chitinase system of *S. plymuthica* has GH18 chitinases, which probably includes exo-acting processive and endo-acting chitinases, and a number of AA10 protein LPMOs, which combinations probably could be used for the efficient degradation of chitin.